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ARF-dependent regulation of ATM and p53 associated KZNF (Apak) protein activity in response to oncogenic stress

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ABSTRACT

The KRAB-type zinc-finger protein Apak (ATM and p53 associated KZNF protein) specifically suppresses p53-mediated apoptosis. Upon DNA damage, Apak is phosphorylated and inhibited by ATM kinase, resulting in p53 activation. However, how Apak is regulated in response to oncogenic stress remains unknown. Here we show that upon oncogene activation, Apak is inhibited in the tumor suppressor ARF-dependent but ATM-independent manner. Oncogene-induced ARF protein directly interacts with Apak and competes with p53 to bind to Apak, resulting in Apak dissociation from p53. Thus, Apak is differentially regulated in the ARF and ATM-dependent manner in response to oncogenic stress and DNA damage, respectively.

Structured summary:
MINT-7989670: *p53* (uniprotkb:P04637) *binds* (MI:0407) to *APAK* (uniprotkb:Q8TAQ5) by *pull down* (MI:0096)
MINT-7989812: *HDM2* (uniprotkb:Q00987) *physically interacts* (MI:0915) with *ARF* (uniprotkb:Q8N726-1) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7989603, MINT-7989626: *APAK* (uniprotkb:Q8TAQ5) *physically interacts* (MI:0915) with *ARF* (uniprotkb:Q8N726-1) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-7989653: *ARF* (uniprotkb:Q8N726-1) *binds* (MI:0407) to *APAK* (uniprotkb:Q8TAQ5) by *pull down* (MI:0096)
MINT-7989686, MINT-7989705, MINT-7989747: *APAK* (uniprotkb:Q8TAQ5) *physically interacts* (MI:0915) with *ARF* (uniprotkb:Q8N726-1) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7989724: *APAK* (uniprotkb:Q8TAQ5) *physically interacts* (MI:0914) with *ARF* (uniprotkb:Q8N726-1) and *p53* (uniprotkb:P04637) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7989635: *ARF* (uniprotkb:Q8N726-1) and *APAK* (uniprotkb:Q8TAQ5) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7989584, MINT-7989773: *APAK* (uniprotkb:Q8TAQ5) *physically interacts* (MI:0915) with *p53* (uniprotkb:P04637) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

The p53 tumor suppressor protein acts as a major defense against cancer. Loss of p53 function occurs during the development of most, if not all, tumor types [1]. p53 functions as a node for integrating whether the cell responds to various types and extents of stress with apoptosis, cell cycle arrest, senescence, DNA repair, cell metabolism, or autophagy [2]. Although the basal level of p53 is low in unstressed cells, p53 can be accumulated and activated in response to a variety of cellular stresses, such as DNA damage, oncogene activation, hypoxia and oxidative stress [3]. The p53 pathway is sensitive to DNA double-strand breaks or single-stranded gaps in the early stage of tumorigenesis. Kinases including ATM (ataxia-telangiectasia mutated) play a crucial part in the immediate response to double-strand breaks through phosphorylation on p53 directly [1]. In response to oncogene activation, p53 is induced through the ARF (alterative reading frame) tumor

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suppressor pathway [4,5] which has been considered to function largely independent of the ATM-mediated DNA damage pathway.

The mammalian p14ARF-p16INK4a locus (p19Arf in the mouse) is frequently mutated in human cancers at an overall frequency of approximately 40% [6–8]. ARF and p16 exist in alternate reading frames with respect to the shared exon 2, and activate the p53 and the Rb tumor suppression pathways, respectively. Mice lacking ARF (homozygous deletion of exon 1β) are prone to tumor development [9], indicating the role of ARF in tumor suppression. Endogenous ARF expression is elevated by oncogenic MYC, Ras, E2F1, E1A and v-Abl, all of which activate p53-mediated cell cycle arrest or apoptosis [10–15].

A few binding partners of p53 have been demonstrated to coordinate the selective regulation of p53 target genes and direct a specific cellular outcome [3]. ASPPs, hCAS/CSE1L, Brn3b, Muc1 and NFκB/p52 selectively activate the expression of apoptotic regulators to promote cell death, whereas Brn3a, Hzf, and YB1 selectively induce p53 activation of genes encoding cell cycle regulators to facilitate cell cycle arrest. We previously identified the KRAB-type zinc-finger (KZNF) protein Apak (ATM and p53 associated KZNF protein) specifically inhibits p53-mediated apoptosis but has no significant effect on the transcription of cell cycle arrest-related genes [16]. Apak interacts directly with p53 through its zinc-fingers and recruits KRAB-box-associated protein-1 (KAP-1) and histone deacetylase 1 (HDAC1) to attenuate the acetylation of p53 through its N-terminal KRAB domain. The inhibitory function of Apak requires the cooperation of ATM kinase. Notably, Apak is required for KAP-1 and ATM regulation of pro-apoptotic but not pro-arrest p53 target genes [16], indicating that the Apak-containing multi-component protein complex might be crucial in determining the p53-mediated cell death. In response to DNA damage, MDM2, p53 and Apak-KAP-1 complex could be sequentially phosphorylated by ATM [17–19]. The Apak phosphorylation on Ser68 by ATM was a late event and caused the dissociation of Apak from p53, allowing efficient p53 activation followed by apoptosis to occur [16,20]. However, whether and how Apak is regulated in response to oncogene activation remains unknown.

In this study, we utilized ectopic expression of c-MYC, E2F1 and Ras-G12V mutant to mimic oncogene activation and investigated the possible regulation of Apak function and the underlying mechanisms. We showed that Apak dissociates from p53 in an ARF-dependent but ATM-independent manner through competitive binding by ARF to Apak. Therefore, these findings deepen our understandings of how the selective p53 regulator Apak was regulated.

2. Materials and methods

2.1. Plasmid constructs and antibodies

Plasmids containing human Apak and p53 were constructed by PCR and recombinant PCR as described previously [16]. Human ARF and a series of deletion mutants were constructed into the *Eco*RI and *Xho*I sites of pCMV-Myc and pCMV-HA vectors (Clontech). For subcellular localization analysis, ARF was subcloned into the *Xho*I and *Bam*HI sites of pDsRed1-N1 vector (Clontech) to express red fluorescence protein (RFP)-tagged fusion protein. Wild-type and mutant c-MYC, E2F1 plasmids are kind gifts from Dr. Pingkun Zhou. Ras-V12 is a kind gift from Dr. Xuemin Zhang. pCMV/HDM2 plasmid was a kind gift from Dr. Yue Xiong and described previously [21]. Specific rabbit polyclonal antibodies recognizing the Ser68-phosphorylated Apak or the unphosphorylated Apak were prepared in our laboratory and described previously [16]. Other antibodies used were anti-ARF (Oncogene), anti-Myc (Clontech), Myc-HRP (Sigma), anti-Flag and anti-Flag-HRP (Sigma), anti-HA (Roche), anti-p53 (Oncogene), anti-p53-HRP (R&D systems), anti-Bax (Santa Cruz), anti-Noxa (Abnova), anti-Fas (Santa Cruz) and anti-HDM2 (Santa Cruz).

2.2. Transfections, cell culture and MMS treatment

HCT116 human colon cancer cells (p53-wild type) were a kind gift from Dr. Qimin Zhan and cultured in DMEM containing 10% FBS. Human lung adenocarcinoma H1299 cells were maintained in RPMI 1640 medium (Hyclone) with 10% FBS. Human embryonic lung HEL cells were a kind gift from Dr. Yi Tie and cultured in DMEM containing 10% FBS. Mammalian cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Methyl methanesulfonate (MMS) was purchased from Sigma and incubated with the cells for 4 h at the concentration of 0.02%.

2.3. Luciferase reporter gene assay

The luciferase reporter plasmid pG13-Luc (pG13L containing 13 tandem p53 binding site repeats) was a kind gift of Dr. Bert Vogelstein. The luciferase reporter assay was performed as described previously [16]. After 48 h transfection, cells were lysed in a passive lysis buffer (Promega). The firefly and renilla luciferase activities were measured with the Dual Luciferase Assay System (Promega) and normalized according to the manufacturer's protocol.

2.4. Immunoprecipitation, immunoblotting, and GST pull-down assays

At 24 h after transfection, cells were treated with the drug for the indicated times and then harvested and lysed in HEPES lysis buffer (20 mM HEPES pH 7.2, 50 mM NaCl, 0.5% Triton X-100, 1 mM NaF, 1 mM dithiothreitol) supplemented with protease inhibitor cocktail (Roche). Immunoprecipitation and immunoblotting, in vitro GST pull-down assays, were performed as we previously described [16,22].

2.5. Cell apoptosis and subcellular localization analysis

Apoptosis assay was performed with Annexin V staining method followed by flow cytometry analysis as described [16]. For subcellular localization analysis, GFP-Apak and RFP-ARF plasmids were transfected into the H1299 cells. Thirty-six hours later, the subcellular localization of Apak and ARF proteins were visualized via a confocal microscope.

2.6. RNA interference

The Apak shRNA sequence (5′-GGGATTATTTGGAAGCCAA-3′) and the control sequence (5′-TGCGTTGCTAGTACCAAC-3′, non-targeting sequence) were cloned into RNAi-Ready pSIREN-DNRDs Red-Express (Clontech) as described [16]. The ARF siRNA (5′-CUCGUGCUGAUGCUACUGA-3′), ATR siRNA (5′-AAGCGCCUGAUUCGAGAUCCU-3′) and non-targeting control siRNA (5′-UUCUCC GAACGUGUCACGU-3′) were synthesised by Shanghai GenePharm. All siRNA transfections were performed with Lipofectamine 2000 (Invitrogen), and the interference efficiency was assessed by western blot.

3. Results and discussion

3.1. Apak dissociates from p53 upon oncogene activation independently of Apak phosphorylation

Since p53 lies at the node of signaling in response to diverse cellular stresses including oncogenic stress and DNA damage, we

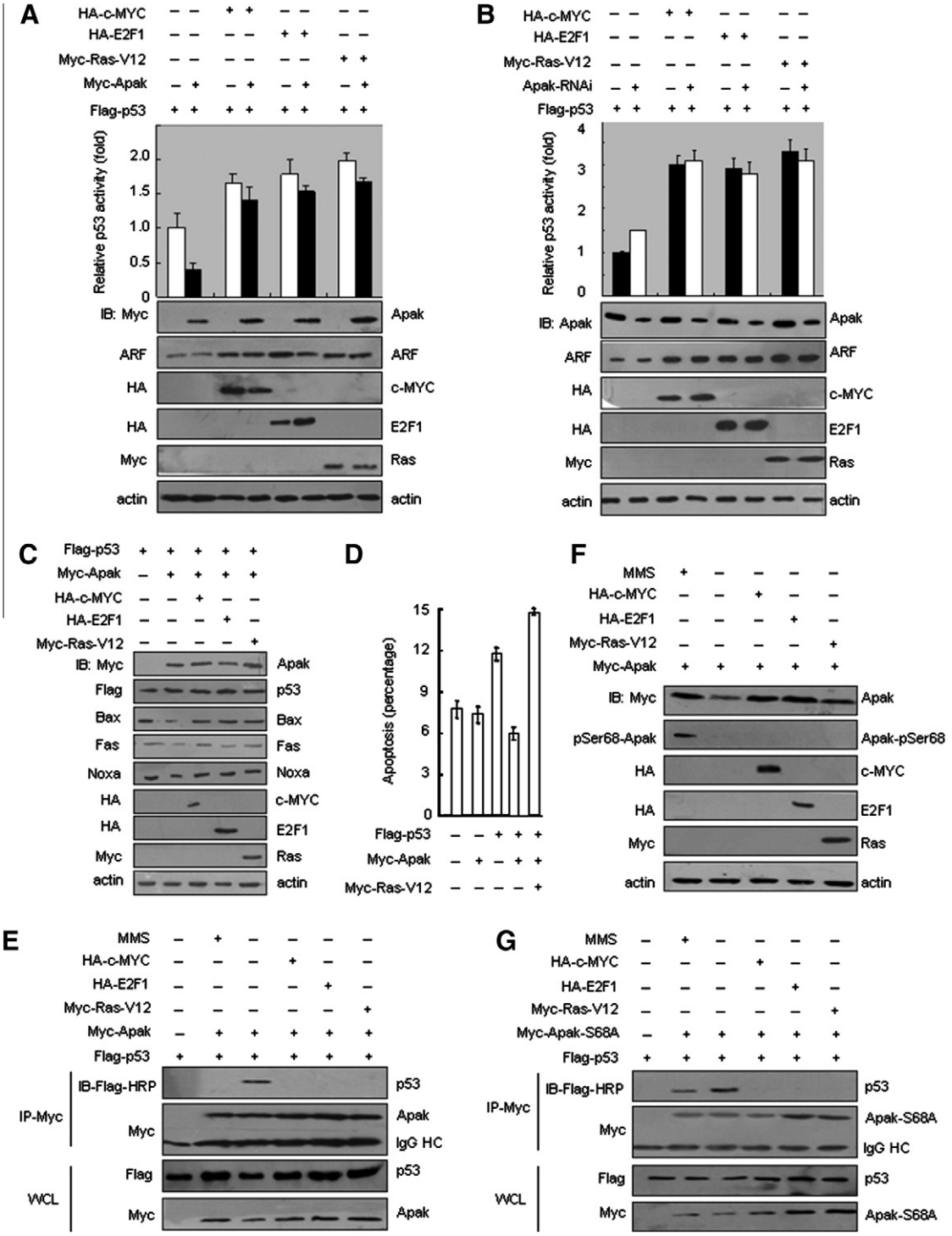

Fig. 1. Apak is negatively regulation upon oncogene activation independent of its phosphorylation. (A) p53-deficient H1299 cells seeded in 24-well plate were transfected with (black) or without (white) Myc-tagged Apak (0.4 μg) in combination with pG13L (0.2 μg), Flag-p53 (30 ng), HA-c-MYC (0.2 μg), HA-E2F1 (0.2 μg), Myc-Ras-V12 (0.2 μg) and control luciferase RL-CMV (1 ng) as indicated. Thirty-six hours after transfection, the luciferase activity was measured and normalized. Representative results of three independent reporter assay experiments are shown. Data are mean ± S.D. (standard deviation, $n = 3$). Western blot analysis of Apak, ARF and oncogene expression was also shown. The antibodies used were indicated on the left and the detected proteins were indicated on the right. IB, immunoblotting. (B) H1299 cells were transfected with or without shRNA-Apak (0.4 μg) in combination with pG13L, Flag-p53, HA-c-MYC, HA-E2F1, Myc-Ras-V12 similar to (A). Thirty-six hours after transfection, the luciferase activity was measured and normalized. Representative results of three independent reporter assay experiments are shown. Western blot analysis of Apak, ARF and oncogene expression was also shown. (C) H1299 cells seeded in 12-well plate were transfected with Myc-Apak (0.6 μg), Flag-p53 (0.4 μg) together with the indicated oncogenes (0.4 μg). Expression of ectopic oncogenes, Apak, p53, and endogenous p53 target genes (Bax, Fas, Noxa) was analyzed by western blotting with the indicated antibodies. (D) H1299 cells in 12-well plate were transfected with Myc-Apak, Flag-p53 together with the Myc-Ras-G12 V as in (C). At 36 h after transfection, cells were collected and analyzed Apoptosis in H1299 cells was determined by staining with Annexin V. Data are mean ± S.D. ($n = 3$).(E) H1299 cells in 25 cm^2 flask were transfected with Myc-Apak (4 μg), Flag-p53 (2 μg) together with the indicated oncogenes (2 μg) or treated with MMS (0.02% for 4 h). The cell lysates were immunoprecipitated with anti-Myc antibody. Both the immunoprecipitate (IP) and the whole cell lysate (WCL) were analyzed by western blot with Myc and Flag antibodies. To avoid the interference of IgG heavy chain, Flag-HRP antibody was used to analysis p53 in IP samples. IgG HC, heavy chain of IgG. (F) H1299 cells in 12-well plate were transfected with Myc-Apak (0.6 μg) and the oncogenes (0.4 μg) as indicated. At 36 h post transfection, cell lysates were analyzed by western blot. MMS treatment was designed as a positive control to indicate the phosphorylation of Apak. (G) Interaction between Myc-Apak-S68A and p53 was abolished by oncogene activation, but not by MMS treatment. H1299 cells were transfected with Apak-S68A and the indicated plasmids. Co-immunoprecipitation assays were performed as in (E).

investigated whether Apak is functionally regulated in response to oncogenic stress mimicked by ectopic expression of c-MYC, E2F1 or Ras-V12, and if it is the case, what is the mechanism. Overexpression of Apak resulted in the remarkable decrease of p53 transcriptional activity (Fig. 1A, column 2 v.s. column 1) whereas overexpression of the c-MYC, E2F1 or Ras-V12 upregulated ARF expression and p53 activity as expected (columns 3, 5 and 7). In these stressed cells, Apak no longer inhibited p53 activity significantly (columns 4, 6, 8 v.s. columns 3, 5, 7, respectively). Depletion of endogenous Apak resulted in an increase of p53 transcriptional activity in the absence of stress whereas it had no significant effect on p53 activity in the presence of oncogenic stress (Fig. 1B). Expression analysis of p53 pro-apoptotic target genes showed that oncogenic stress reversed the inhibitory effects of Apak on p53 transcriptional activation (Fig. 1C). Apak inhibited the p53-induced cell apoptosis (Fig. 1D, column 4 v.s. column 3) and Ras-V12 ectopic expression significantly reversed the effect of Apak (column 5 v.s. column 4). These results indicated that Apak was functionally inhibited in response to oncogene activation.

Co-immunoprecipitation assays showed that like the treatment with DNA damaging agent methyl methanesulfonate (MMS), expression of c-MYC, E2F1 or Ras-V12 induced Apak dissociation from p53 (Fig. 1E). We previously demonstrated that MMS treatment triggers Apak phosphorylation on Ser68 by ATM kinase and this phosphorylation is both sufficient and necessary for DNA damage-induced Apak-p53 dissociation [16,20]. We then ask whether the dissociation of Apak from p53 in response to oncogenic stress is similar to or distinct from the case in response to DNA damage stress. Direct western blot analysis with the anti-phospho-Apak-Ser68 antibody did not detected any band in c-MYC, E2F1 and Ras-V12-expressing cells, although the total Apak protein level was upregulated compared with the unstressed cells (Fig. 1F, lanes 3-5). As a positive control, Apak phosphorylation could be easily detected in MMS-treated cells (lane 1). Substitution of Ser68 with alanine had no effect on the dissociation of Apak from p53 in response to oncogene activation but blocked their dissociation in response to MMS treatment (Fig. 1G). These data suggested that the negative regulation of Apak activity in response to oncogene activation was independent on Apak phosphorylation, at least the ATM kinase-mediated phosphorylation on Ser68.

3.2. Apak is negatively regulated dependently of ARF upon oncogene activation

It has been well-accepted that in response to oncogene activation, p53 is induced and activated largely through the ARF tumor suppressor pathway [4,5]. We then examined whether the negative regulation of Apak function is dependent on ARF. Knockdown of endogenous ARF by RNA interference (RNAi) but not control small interfering RNA (siRNA) transfection blocked the negative regulation of Apak activity by oncogene activation (Fig. 2A). ARF knockdown also prevented the oncogene-induced dissociation between Apak and p53 and then inhibited p53 accumulation (Fig. 2B). Similar results were obtained in H1299 cancer cells (Fig. 2A and B) and primary HEL (human embryonic lung) cells (Supplementary Fig. S1A and B). Thus, oncogene activation regulated Apak activity and the Apak-p53 interaction dependently of ARF.

Human ARF protein consists of 132 amino acids, among which the N-terminal 64 residues are encoded by the unique exon 1β [6,7]. Overexpression of the ARF N-terminal half alone is sufficient to activate p53. The N-terminal half possesses most of the known binding abilities and has been suggested to play a pivotal role in the tumor suppressor activity of ARF. Until recently, the C-terminal half was shown to bind to p32 and critical for ARF to localize to mitochondria and induce apoptosis [23]. We next determined the functional region of ARF required for Apak regulation. Coexpression of ARF with Apak completely blocked the repressive activity of Apak on p53 (Fig. 2C, column 3). Overexpression of the ARF N-terminal half (N64) alone was sufficient to inhibit Apak (column 4), whereas the ARF C-terminal half (ΔN64) had no any effect on Apak function (column 5). Further deletion analysis revealed that the region comprising of aa 46–64 was required for ARF to inhibit Apak activity (columns 6–8). Previous studies have identified ATR to be critical for ARF-mediated activation of p53 [24]. To examine whether ATR is involved in ARF-mediated inactivation of Apak, endogenous ATR was depleted by RNAi and the regulatory effect of ARF on Apak function was analyzed. Fig. 2D showed that ATR depletion had weak effects on the ARF-mediated inactivation of Apak, suggesting that ARF inhibited Apak activity largely independent on ATR.

3.3. ARF interacts with Apak through the central region of aa 46-64

We next tested whether ARF inhibited Apak through direct binding. In vitro GST pull-down assays showed that both the full-length ARF (GST-ARF WT, lane 3) and the N-terminal ARF (GST-ARF N64, lane 4) could strongly bind to His-tagged Apak but the C-terminal ARF (GST-ARF ΔN64, lane 5) or GST alone (lane 1) could not (Fig. 3A). As a positive control, Apak could interact with p53 as we previously reported (lane 6) [16]. To confirm the interaction between ARF and Apak in vivo, co-immunoprecipitation assays were performed in p53-deficient H1299 cells since endogenous ARF and Apak expression is higher in such cells than the p53-wild type cells. As shown in Fig. 3B, ARF was clearly detected in the immunoprecipitates obtained with the anti-Apak antibody but not the pre-immune serum. Conversely, endogenous Apak was readily immunoprecipitated with the ARF-specific antibody which specifically recognized the N-terminal part of ARF (Supplementary Fig. S2) but not a control IgG. Importantly, even in p53-wild type cells with low level of endogenous ARF level, ARF could be induced by ectopic Ras-V12 expression and interactions between endogenous ARF and Apak could be detected (Fig. 3C), indicating that the ARF-Apak interaction could be induced or enhanced in response to oncogene activation. It has been well-defined that ARF resides predominantly in the nucleolus. We previously showed that Apak protein is localized predominantly in the nucleoplasm and less to the nucleolus [16]. When coexpressed with exogenous ARF, Apak was colocalized with ARF in the nucleoli (Fig. 3D). These data indicate that ARF and Apak interact with each other both in vitro and in vivo.

A series of ARF and Apak deletion mutants were generated and used for mapping their interacting regions. The N-terminal ARF comprising of aa 46–64 was required for Apak binding, whereas either the 1–45 or 46–64 region could mediate the HDM2-binding (Fig. 3E). The latter result is consistent with a previous study which identified multiple regions within 1–64 of ARF-mediated MDM2 interaction and inhibition [25]. Our data indicated that the Apak-binding and HDM2-binding regions on ARF were partially overlapped but not conflicted. For Apak, the zinc-fingers of Apak mediated its interaction with ARF (Fig. 3F). Among the nine truncates of Apak we examined, only the N1 (KRAB domain only) mutant was unable to interact with ARF, suggesting that four zinc fingers are sufficient for ARF binding. Since the nineteen zinc fingers of Apak are highly conserved in their sequences, the position of the zinc fingers seem to be irrelevant to the ability to bind to ARF.

3.4. ARF competes with p53 to bind to Apak

Interestingly, we previously showed that Apak interacts with p53 through its zinc fingers. This similar pattern prompted us to

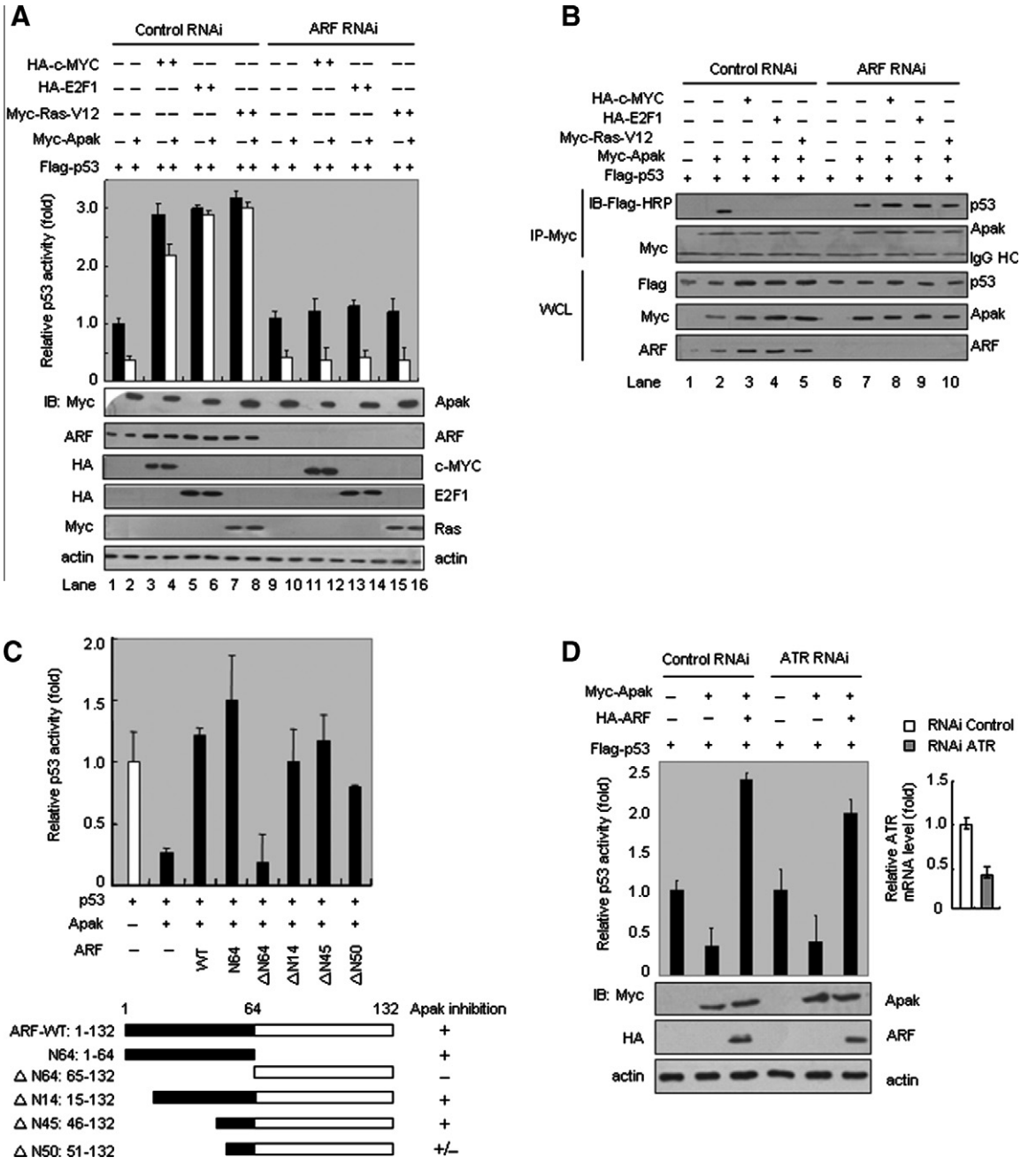

Fig. 2. ARF is required for the negative regulation of Apak by oncogenic stress. (A) H1299 cells in 24-well plate were transfected with pG13L (0.1 μg), Flag-p53 (30 ng), Myc-Apak (0.3 μg), the oncogenes (0.2 μg), control luciferase RL-CMV (1 ng), and ARF siRNA or control siRNA (0.2 μg) as indicated. At 36 h post transfection, p53 activity was measured by reporter gene assays. Data are mean ± S.D. (n = 3). The efficiency of ARF knockdown was determined by western blot. (B) H1299 cells were transfected with the indicated plasmids and siRNAs. The cell lysates were immunoprecipitated with anti-Myc antibody to precipitate Apak proteins. The bound p53 was detected by western blot. (C) H1299 cells were transfected with pG13L (0.2 μg), Flag-p53 (30 ng), Myc-Apak (0.4 μg), and the indicated ARF deletion mutants (0.2 μg). At 36 h post transfection, p53 activity was measured by reporter gene assays. (D) H1299 cells were transfected with pG13L (0.2 μg), RL-CMV (1 ng), Flag-p53 (30 ng), Myc-Apak (0.3 μg), ARF (0.1 μg), and ATR siRNA or control siRNA (0.2 μg) as indicated. At 36 h post transfection, p53 activity was measured by reporter gene assays. Data are mean ± S.D. (n = 3). Apak and ARF were analyzed by western blotting. The efficiency of ATR knockdown was determined by qPCR. Data are mean ± S.D. (n = 3).

speculate whether oncogene upregulated ARF proteins could compete with p53 to bind to Apak. Apak was coexpressed with exogenous p53 together gradually increased amounts of ARF. The Apak proteins were immunoprecipitated and the bound p53 and/or ARF proteins were detected by western blot. As shown in Fig. 4A (lanes 1–5), the gradual increased expression of ARF significantly enhanced the interaction between Apak and ARF but reduced the interaction between Apak and p53. Reciprocal experiments showed that p53 could also compete with ARF to bind to Apak (lanes 6–9). The competition was dependent on the direct binding between ARF and Apak due to the fact that ARF deleted of 46–64 residues lost the ability to compete with p53 because it could no longer interact with Apak (lanes 10–14). Based on the fact that endogenous ARF was elevated in response to oncogene activation, we proposed that the elevated ARF competed with p53 to bind to Apak, therefore disrupted the Apak-p53 interaction.

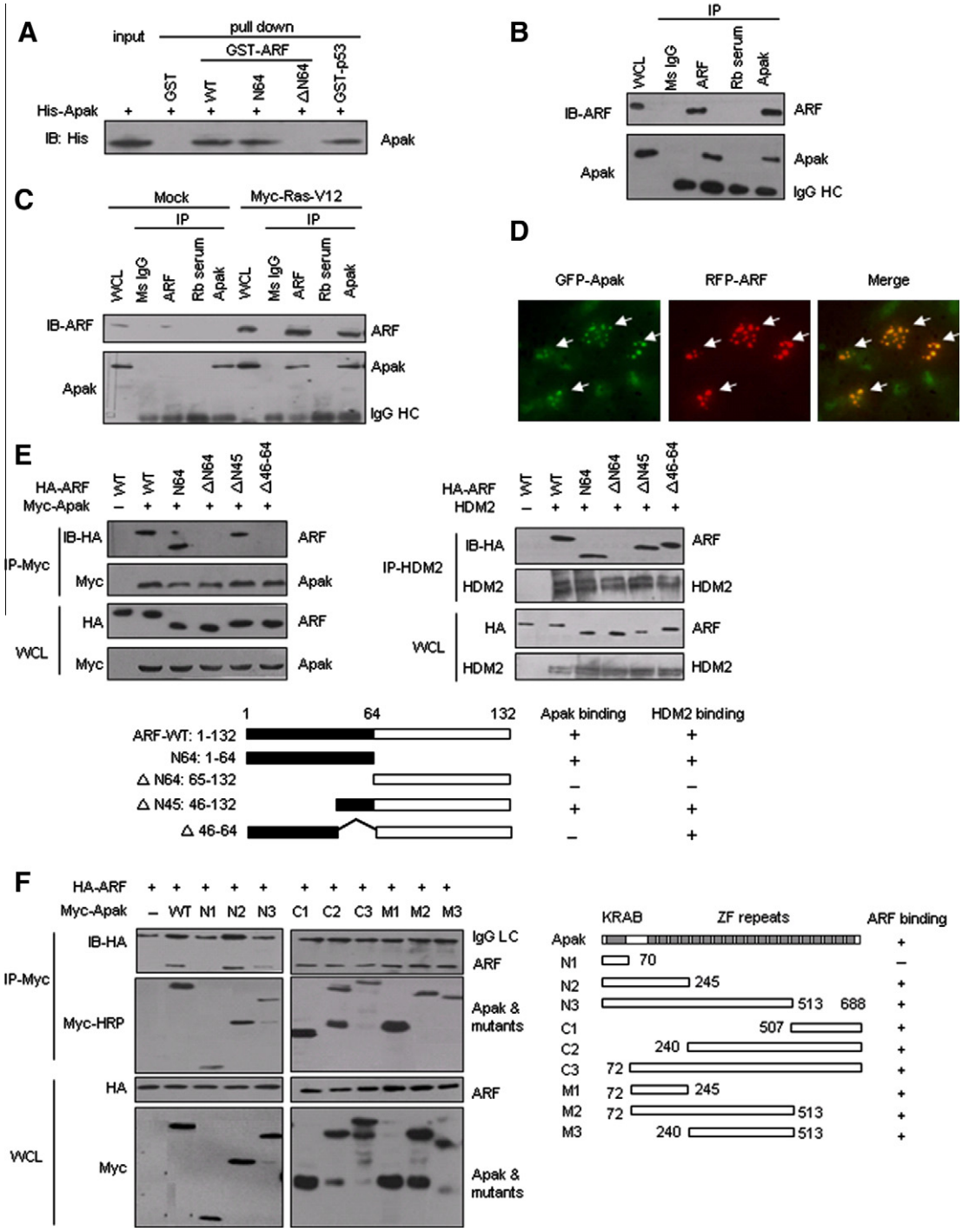

Fig. 3. Apak interacts with ARF both in vitro and in vivo. (A) Direct interaction between Apak and ARF revealed by GST pull-down assays. The wild-type GST-ARF full-length protein, the mutants GST-ARF N64 and ΔN64, the positive control GST-p53, or GST alone was used in the assay with His-Apak. Both input and pull-down samples were subjected to immunoblotting with anti-His and anti-GST antibodies. Input represents 10% of that used for pull-down. (B) Co-immunoprecipitation of endogenous Apak and endogenous ARF from H1299 cells. Western blot analysis of whole cell lysate (WCL) and immunoprecipitation (IP) with Apak-specific antibody, ARF antibody, or control IgG. (C) Induced interaction between Apak and ARF in response to oncogene activation. $p53^{+/+}$ HCT116 cells were transfected with Ras-V12 and endogenous interaction between Apak and ARF was detected. Both the WCL and IP samples were subjected to western blot analysis. IgG HC, heavy chain of IgG. (D) Co-localization of Apak and ARF in the nucleoli of H1299 cells. GFP-tagged Apak and RFP-tagged ARF were co-transfected into H1299 cells. Images were captured by a confocal microscope at 36 h post transfection. The arrows indicate ARF- and Apak-coexpressing cells. (E) (left) Mapping the region of ARF that interacts with Apak. Full-length or truncates of ARF were co-transfected with Myc-Apak into HCT116 cells. Equal amounts of cell extract were IP with anti-Myc antibody, and analyzed by Western blot with anti-HA antibody. Both the WCL and immunoprecipitates were analyzed. (right) Mapping the region of ARF that interacts with HDM2. Full-length or truncates of ARF were co-transfected with HDM2 into HCT116 cells. Equal amounts of cell extract were IP with anti-HDM2 antibody, and analyzed by Western blot with anti-HA antibody. Both the WCL and immunoprecipitates were analyzed. (F) Mapping the region of Apak that interacts with ARF. Full-length or truncates of Apak and HA-ARF were transfected into HCT116 cells. Equal amounts of cell extract were immunoprecipitated with anti-Myc antibody, and analyzed by Western blot with anti-HA antibody. Both the WCL and immunoprecipitates were analyzed. IgG LC, light chain of IgG.

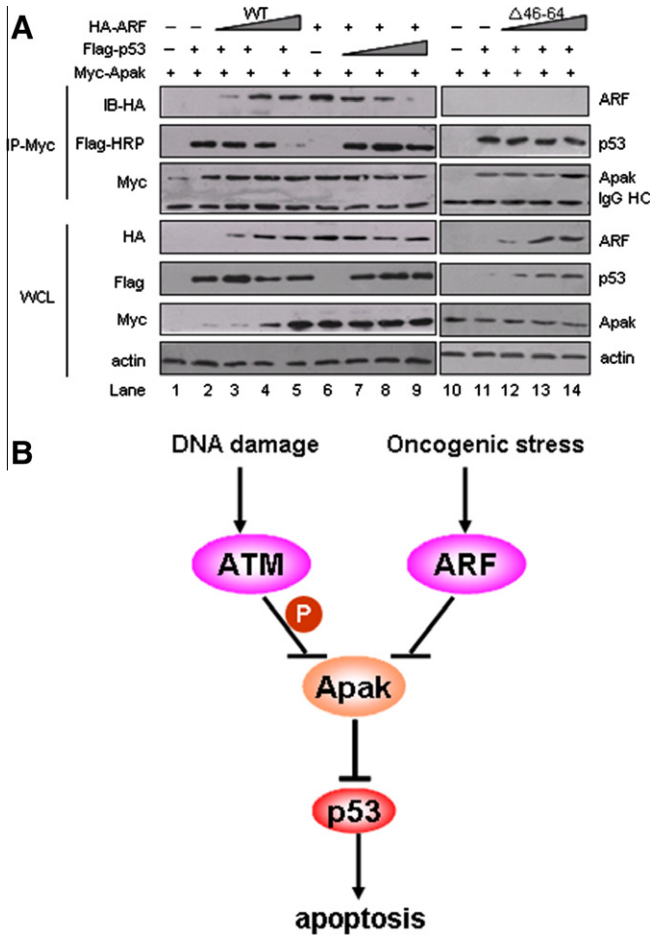

Fig. 4. ARF competes with p53 to bind to Apak. (A) H1299 cells were transfected with the increased amounts (1 μg, 2 μg, 4 μg) of HA-ARF (wild type or the Δ46–64 mutant), Flag-p53 (2 μg) and Myc-Apak (3 μg) or transfected with the increased amounts (1, 2, 4 μg) of Flag-p53, HA-ARF (2 μg) and Myc-Apak (3 μg). Immunoprecipitations were performed with anti-Myc antibodies; the lysates and the immunoprecipitates were analyzed by western blot with the indicated antibodies. (B) Apak is negatively regulated by DNA damage and oncogenic stress in the ATM- and ARF-dependent manner, respectively.

Taken together, Apak is negatively regulated in response to DNA damage and oncogene activation in the ATM- and ARF-dependent manner, respectively (Fig. 4B). Based on the competitive binding model, the balance between the abundances of ARF and p53 should determine the switch of Apak-containing protein complex. Under unstressed conditions, both ARF and p53 levels are kept low. Oncogene activation induces the new synthesis of ARF, which has been suggested to be driven by the transcription factor E2F1 [10]. Then the elevated ARF proteins overcome the low level of p53 proteins to bind to Apak and disrupt the Apak-p53 interaction. We also speculate that after p53 was stabilized and activated, the p53 proteins with higher stability might contrariwise compete with ARF to bind to Apak and are re-inhibited by Apak, preventing the improper overactivation of p53.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.08.015.

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